# Fluorescent Labeling of Total RNA **Updated 01/28/04**

## 1. Add primer to the RNA samples to be labeled.

- Make up fresh or thaw previously made Master Mixes B. (See Master Mix Table)

Prime the two RNA samples to be compared

| | |
|---|---|
| RNA sample (30 µg total RNA per rxn) in $dH_2O$ | 15 µl |
| Oligo dT primer (1mg/ml) | 1 µl |
| Total | 16 µl |

(See notes 1,2,6)

## 2. Heat and snap cool.

- Heat samples to 70°C for 5 min., then snap cool in an ice-water bath. Centrifuge briefly to collect the contents to the bottom of the tube.

## 3. Initiate two labeling reactions, one for Cy3 and one for Cy5.

Add these components in the order listed

| | |
|---|---|
| RNase inhibitor diluted 2X | 0.5 µl |
| Master Mix B | 10.0 µl |
| dUTP-Cy3 **OR** dUTP Cy5 | 2.0 µl |
| Superscript II | 1.5 µl |

Total Volume should now be 30ul
(See Note 3)

- Mix the components well with the RNA/primer sample and centrifuge briefly to collect the contents to the bottom of the tube

## 4. Incubate reverse transcription reaction mixture at 42°C for 90 min

## 5. Degrade RNA

- After the reverse transcription reaction is complete, add 10µl of 1M NaOH and heat at 70°C for 15 min.
- Neutralize with 10µl 1M HCl.

## 6. Clean up labeled cDNA

- Combine the appropriate Cy3- and Cy5-labeled samples that will be co-hybridized onto the array.
- Add the combined probes to a Vivaspin 500 (10K cutoff) with 400 µl TE. Centrifuge at 12,000 X g (~10,000 rpm) for 8 min. Discard flow through.
- Repeat the wash step in the same Vivaspin filter at least three times.
- We recommend 2-3 additional washes in a fresh Vivaspin 500. Add 400 µl TE, centrifuge at 12,000 X g (~10,000 rpm) for 8 min. Discard flow through.
- Remove the sample retained in the filter chamber with a pipette and transfer to a new tube. Adjust the sample volume to 20 µl for the mouse and human standard microarray formats.

**Notes**

**Note 1**. This protocol is designed to produce two cDNA samples in parallel that are labeled with different fluorescent dyes – one with Cy3 and the other with Cy5. Total RNA is the template for the reverse transcription reaction that creates the cDNA copy and directly incorporates a fluorescent nucleotide (either Cy3-dUTP or Cy5-dUTP). These two differentially labeled cDNA pools are combined in Step 6 and co-purified to remove excess nucleotides, primers, salts and buffers. Adequate purification of the labeled cDNA is essential to reduce hybridization background fluorescence. We have found that labeling of 30µg RNA is appropriate for both Cy3 and Cy5.

**Note 2.** Keep the RNA and other reaction components on ice until instructed otherwise. Use RNase-free reagents and technique throughout the labeling procedure. The best results are typically generated by high-quality, pure RNA preparations. Users are encouraged to check the integrity and quantity of their RNA before labeling.

**Note 3.** Use of the RNase inhibitor is optional.

**Master Mix B Table**

| Reagent | Stock concentration | Volume for 1X (Assumes 30µl reaction volume ) | Bulk mixture for 100 reactions |
|---|---|---|---|
| 5X RT buffer (Invitrogen) | 5X | 6 µl | 600 µl |
| 100 mM DTT | 100 mM | 3 µl | 300 µl |
| dATP | 100 mM | 0.15 µl | 15 µl |
| dCTP | 100 mM | 0.15 µl | 15 µl |
| dGTP | 100 mM | 0.15 µl | 15 µl |
| dTTP | 100 mM | 0.03 µl | 3 µl |
| Water (RNase-free) | | 0.52 µl | 52 µl |

Master Mix B should be made up in sufficient volume to allow for accurate pipetting of each component. Master Mix B may be stored frozen. Distribute Master Mix B into aliquots to minimize the freeze-thaw cycles. Avoid thawing a Master Mix B tube more than twice after it is made. Thaw or create enough Master Mix solution in sufficient quantity to complete all reactions plus one

**Note 4**. Researchers must keep accurate notes on which samples are labeled with which fluorescent dyes. The table below defines some of the properties of each dye.

| Cyanine dye | Cy3 | Cy5 |
|---|---|---|
| Visual color of dye | Pink | Blue |
| Excitation maximum | 550 nm | 650 nm |
| Emission maximum | 568 nm | 668 nm |
| Extinction coefficient ($M^{-1}cm^{-1}$) | 150000 | 250000 |
| Axon scanner channel | 532 nm | 635 nm |
| Standard pseudo colored display (by the software) | Green | Red |
| Vulnerabilities | Higher background than Cy5 | Quenched by dense packing; more labile on dry slides |

# Appendix

Table A-1. Sources of materials and reagents for microarray labeling and hybridization

| Vendor | Item | Catalog |
|---|---|---|
| Ambion<br>www.ambion.com<br>800-888-8804 | Superase In (RNase inhibitor) | 2694 |
| Amersham Pharmacia<br>www.apbiotech.com<br>800-526-3593 | Cy3-dUTP, 25nmol, quote 752BQ | PA53022 |
| | Cy5-dUTP, 25nmol, quote 752BQ | PA55022 |
| | dNTP set (dATP, dCTP, dGTP, dTTP) 4X25umol | 27-2035-01 |
| | Random haxamer pdN(6) (for prokaryotic RNA) | 27-2166-01 |
| Invitrogen ,<br>www.invitrogen.com,<br>888-584-8929, BPA# 263-00040377 *** | Superscript II reverse transciptase, RNaseH-neg | 18064014 |
| | Oligo dT primer (12-20mer) 1 mg/ml | Poly.gf |
| | 100mM dNTP set, 4X 25 µmol | 10297-018 |
| Vivascience<br>www.vivascience.com | Vivaspin 0.5ml Concentrator | VS0102 |

* Must reference quote # 752BQ for NIH discount from Amersham Pharmacia through 12/31/04.
** May require lead time, minimum order 5oz
*** BPA pricing is less than catalog listing in table

This table lists materials and products that will perform adequately in this procedure. However, this table does not represent an endorsement of any company or product. There are other products that can be substituted for the items listed with no adverse consequence on the results.